Aquaculture 550 (2022) 737844

Gonadotropin induction of spermiation in Senegalese sole: effect of temperature and stripping time

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Highlights

- Treatment with rFsh and rLh at 12ºC enhance spermiation in Senegalese sole F1 males.
- One batch of spermatids is recruited into spermatozoa differentiation after a single rLh injection.
- Maximum sperm production occurs 48 h after rLh injection at 12ºC.
- rFsh and rLh treatments at 12ºC and 17ºC, respectively, increase spermiation.

Abstract

Treatments with homologous recombinant follicle-stimulating and luteinizing hormones (rFsh and rLh, respectively) are known to enhance spermatogenesis and sperm production in sole, but the response can be highly variable depending on the dose, duration and time of the year of the rFsh treatment. To further investigate the physiological effects of rFsh and rLh on sperm production in sole, here we examined the pattern of spermiation of F1 males, of approximately 450 g, treated with rFsh and rLh under controlled temperature. In an initial trial at 12ºC, males were weekly injected intramuscularly with 18 µg kg^{-1} rFsh over five weeks and subsequently treated with a single injection of 18 µg kg^{-1} rLh. Histological analysis indicated that the rFsh+rLh treatment increased gonad weight and stimulated spermatogenesis, and also enlarged the size of the seminiferous and efferent duct (ED) tubules, resulting in a doubling of sperm production with respect to the controls. Sperm counts in the ED and sequential stripping of males at 24, 48 and 72 h post rLh injection further revealed that only one batch of spermatids is recruited into spermatozoa (Spz) differentiation after a single rLh induction. A peak of sperm accumulation in the ED occurs at 48 h, coinciding with the upregulation of genes potentially involved in Spz maturation. In a second experiment, we tested the effect of two rFsh doses (10 or 18 µg kg^{-1}) over five weeks as previously, followed by one rLh injection at 12ºC or 17ºC. The results confirmed that spermiation was the highest 48 h after rLh treatment at 12ºC, which was increased in a dose-dependent manner with the dose of rFsh previously supplied (from 0.36 to 0.95 x 10^9 Spz kg^{-1}). However, sperm production elicited with the low rFsh dose was potentiated by ~3-fold (from 0.36 to 1.06 x 10^9 Spz kg^{-1}) when the rLh treatment was given at 17ºC. These data suggest that in Senegalese sole sperm collection should be carried out at 48 h after rLh treatment, and that a low dose of rFsh at 12ºC is highly efficient for stimulating sperm production when rLh is administered at a temperature close to that occurring during maximum natural spermiation.

Keywords

Flatfish, Recombinant gonadotropins, Spermatogenesis, Spermiation, Temperature

1. Introduction

The control of reproduction in aquaculture is critical to provide good quality gametes for the mass production of larvae, and to facilitate methods to preserve traits of commercial interest through genetic breeding programs (Lind et al., 2012). In the last decades, the high demand for the diversification of marine fish aquaculture has identified the Senegalese sole (*Solea senegalensis*) as one of the target species in the Southern Mediterranean because of its high commercial value (FAO, 2018). However, the domestication of this species to assure the sustainability of its culture is impaired by the lack of methods to control reproduction, particularly of the F1 offspring of wild captive broodstock, which results in the obtention of poor or none fertilization, as well as variable larval quality and high incidence of abnormalities, heterogenous growth or mortality (Morais et al., 2016). As for other species (Mylonas et al., 2017), the use of *in vitro* fertilization in Senegalese sole culture has been proposed as a more controlled method for obtaining eggs and larvae (Liu et al., 2008; Rasines et al. 2012ab; Ramos-Júdez et al., 2021b). However, the low quantity and variable quality of the sperm that the sole males typically produce (Beirão et al., 2011; Cabrita et al, 2011) impedes the transfer of these protocols to the industry.

The Senegalese sole is oligospermic (producing <130 µl of semen), as other flatfishes, and shows asynchronous and semicystic spermatogenesis, i.e. the differentiation of haploid spermatids to spermatozoa (spermiogenesis) takes place within the lumen of the seminiferous tubules (García-López et al., 2005). Due to the asynchronous nature of sole spermatogenesis consecutive batches of spermatids are recruited into spermatozoa differentiation during the year, and consequently spermiation occurs all year-round. However, sperm production is more intense during spring, when females ovulate, which coincides with a peak in the plasma levels of the gonadotropins follicle-stimulating (Fsh) and luteinizing (Lh) hormones and of the major androgen 11-ketotestosterone (11-KT) (García-López et al., 2006; Cabrita et al., 2011; Chauvigné et al., 2015, 2016). During the last years, different hormone treatments based on the administration of gonadotropin-releasing hormone analogue (GnRHa) or human chorionic gonadotropin, with or without 11-KT precursors, such as 11-ketoandrostenedione, or dopaminergic inhibitors, have been tested with the aim of increasing semen production in Senegalese sole. However, none of these treatments result in a marked increase of spermiation, although they do induce a transient elevation of circulating androgens, and may increase the hydration or the motility of sperm (Agulleiro et al., 2006, 2007; Cabrita et al., 2011; Guzmán et al., 2011ab).

Recently, however, the use of Senegalese sole recombinant Fsh and Lh (rFsh and rLh, respectively), which activate specific receptors in somatic and germ cells in the testis, have shown to be useful to enhance sperm production. Recombinant gonadotropins can be produced as single-chain polypeptides in different heterologous host systems, such as the yeast or mammalian cells, which allows continuous availability of the hormones (Dalton and Balton, 2014; Molés et al., 2020). Treatment with recombinant gonadotropins is effective at inducing spawning and spermiation in several fish species (Sanchís-Benlloch et al., 2017; Zhang et al., 2018; Peñaranda et al., 2018; Kobayashi et al., 2010; Mazón et al, 2013, 2014; Molés et al., 2020, Ramos-Júdez et al., 2021a), which highlights the great potential of these hormones for aquaculture. In Senegalese sole, homologous rFsh and rLh can stimulate spermatogenesis and spermiogenesis *in vitro* (Chauvigné et al., 2012, 2014ab), as well as increase testicular growth, spermatogenesis and spermiation *in vivo* (Chauvigné et al., 2017, 2018). However, these treatments can sometimes produce results with a high variability, which

may be related to the duration and dose of the rFsh treatment and the time of the year when this hormone is administered (Chauvigné et al., 2017, 2018). In addition, the time-course effects of rLh on spermiation *in vivo*, which are crucial in order to select the best time for the collection of mature and highly motile sperm, are not known. Therefore, to establish reliable recombinant gonadotropin-based hormone therapies for increasing semen production in the Senegalese sole it is necessary to decipher the physiological effects of rFsh and rLh on spermatogenesis and spermiation.

In the present study, we have examined the production of sperm by pubescent sole F1 males after treatment with increasing doses of rFsh at low temperature, and subsequent induction of spermiation with rLh at low and high temperatures. In addition, by sequential or separate stripping of males and histological analysis we have investigated the pattern of sperm production at different times after rLh treatment. These new data and approaches provide a significant advance towards the establishment of industrial protocols for spermiation enhancement in Senegalese sole.

2. Materials and methods

2.1. Animals and recombinant hormones

The fish employed in this study were approximately two-year pubescent Senegalese sole F1 males, which were maintained at the Institute of Agrifood Research and Technology (IRTA) research facilities in Sant Carles de la Ràpita (Spain), as previously described (Chauvigné et al., 2017), or at the facilities of Safiestela-Sustainable Aqua Farming Investments in Porto (Portugal). The experimental procedures relating to the care and use of animals were approved by the Ethics Committee from IRTA and the Portuguese legislation for the use of laboratory animals in accordance with the guidelines of the European Directive (2010/63/EU).

Single-chain Senegalese sole rFsh and rLh were produced in Chinese hamster ovary (CHO) cells by Rara Avis Biotec (Valencia, Spain) as described previously (Chauvigné et al., 2017). The biological activity of the hormones produced for the present study was confirmed by intramuscular injection of male fish and measurement of 11-KT plasma levels at 48 h after injection (see below).

2.2. Experimental design

2.2.1. Experiment 1

Males (394 ± 12 g; mean ± SEM) were kept in 10 m^3 tanks connected to a recirculation system (IRTAmar1) and acclimated to 12°C for 2 weeks (from late October to mid-November) under a natural photoperiod. Based on previous studies (Chauvigné et al., 2018), fish were injected intramuscularly with a dose of 18 µg kg^{-1} of rFsh (n = 25) or saline buffer (controls, n = 25) once a week for 5 consecutive weeks. One week after the last injection, only fish treated with rFsh were injected with a single dose of rLh (18 µg kg^{-1}), while control males were treated again with saline. Ten fish from each group were sequentially stripped at 24, 48 and 72 h after rLh treatment, whereas other 5 fish were sacrificed at each time. Blood samples were taken before the first injection with rFsh (time 0) as well as at 24, 48 and 72 h after rLh treatment.

2.2.2. Experiment 2

Fish (517 ± 14 g) were acclimated to 12°C during approximately four months (from October to mid-February) with a photoperiod of 10 h light:14 h dark. After this

period, fish were divided into the following experimental treatments: Groups 1 and 2 (*n* = 12 each) were injected with saline; Groups 3 and 4 (*n* =12 each) were treated with 10 µg kg^{-1} rFsh; and Groups 5 and 6 (*n* = 12 and 36, respectively) were injected with18 µg kg^{-1} rFsh. These treatments were administered for 5 consecutive weeks. After this time Groups 2, 4 and 5 were acclimated to 17ºC for one week, to test the effect of temperature on hormone-induced spermiation, whereas Groups 1, 3 and 6 remained at 12ºC. Fish from all groups including the controls were then injected with 18 µg kg^{-1} rLh. Sperm was stripped at 48 h after rLh treatments in Groups 1 to 5, whereas the males from Group 6 were divided into three groups (*n* = 12 each) that were stripped for sperm collection at 24, 48 or 72 h. Blood samples were taken before the start of the experiment (time 0), the day before rLh injection at 12ºC (day 42) and during the following three days (24, 48 and 72 h, days 43, 44 and 45, respectively). For the fish treated with rLh at 17ºC (Groups 2, 4 and 5) plasma samples were collected after temperature acclimation to 17ºC prior to rLh injection and two days after injection (48 h, day 44).

2.3. Sampling procedures

Sperm and blood samples were collected as previously described (Chauvigné et al., 2017). For the extraction of testis biopsies, fish were sedated before being sacrificed by decapitation and the entire testis removed in order to determine the gonadosomatic index (GSI; testes weight fish $weight^{-1}$ x 100). The dorsal testis was fixed in Bouin's solution (5% acetic acid, 9% formaldehyde, and 1.5% picric acid in aqueous solution) overnight at room temperature for further histological analysis. The left testis was cut into two pieces that were deep frozen in liquid nitrogen and kept at -80°C for subsequent gene expression analysis.

2.4. Gonadotropin and steroid determinations

To determine plasma levels of both endogenous and recombinant gonadotropins enzyme-linked immunosorbent assays (ELISAs) using specific antibodies against Senegalese sole Fshβ and Lhβ subunits were carried out following established protocols (Chauvigné et al., 2015, 2016). A commercial enzyme immunosorbent assay (EIA; Cayman Chemical Company) was used to determine 11-KT levels in plasma as previously described (Chauvigné et al., 2015, 2016, 2017). Plasma free steroids were extracted in methanol from 3.5 µl of plasma and the resulting pellet was diluted 1:100 in EIA buffer 0.1M K_2HPO_4/KH_2PO_4, 1.54 mM sodium azide, 0.4M NaCl, 1 mM EDTA, and 0.1% BSA, pH 7.4). A standard curve was run for each EIA plate and all samples were analysed in duplicate.

2.5. Histological analysis

Testis fixed in Bouin's solution were subsequently dehydrated and embedded in paraplast (Sigma-Aldrich). The testis biopsies were oriented in the molds in a manner to obtain sagittal sections. Sections of 7 µm in thickness were attached to UltraStick/UltraFrost Adhesion slides (Electron Microscopy Sciences) and stained with hematoxylin and eosin as previously described (Chauvigné et al., 2017). The different somatic and germ cell types in the Senegalese sole testis were identified following the descriptions by García-López et al. (2005). The relative amounts (%) of spermatogonia type A and B (SpgA and SpgB, respectively), spermatocytes (Spc), attached and free spermatids (Spd_A and Spd_F, respectively), and spermatozoa (Spz) were scored in 10 tubules from different testicular areas per fish. The area of the tubules of the efferent

duct and the number of spermatozoa in each tubule were also scored in 3 representative tubules per fish. Counting of the cell types in the testis and efferent duct tubules was carried out in 5 different fish for each group at each time point using the NIS-element AR 4.30.02 software (Nikon).

2.6. RNA extraction and gene expression analysis

The expression levels of selected genes, such as sperm antigen 6 (*spag6*), sperm surface protein 17 (*spa17*), cilia- and flagella-associated protein 46, 54 and 61 (*cfap46*, *cfap54*, *cfap61*, respectively), radial spoke head protein 1 (*rshp1*), cytochrome P450 family 17 subfamily A member 1 and 2 (*cyp17a1* and *cyp17a2*, respectively), 20β-hydroxysteroid dehydrogenase (*cbr1*), and membrane progestin receptor alpha (*paqr7*), were determined by real-time quantitative RT-PCR (qRT-PCR). Total RNA was extracted from the testes using the GenElute^{TM} Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), treated with DNase I, and 1 µg of total RNA was reverse transcribed using 0.5 µg oligo (dT)17, 1 mM dNTPs, 40 IU RNAse inhibitor, and 10 IU SuperScript II (Life technologies Corp.) for 1.5 h at 42°C. The qRT-PCR was carried out in a final volume of 20 µl using 5 µl of SYBR Green qPCR master mix (Life Technologies Corp.), 1 µl of diluted cDNA (1:5 in sterile mQ water), and 0.5 µM of each forward and reverse primer (Table 1). The reference gene was alpha actin (Table 1). Each sample was assayed in duplicate on 384-well plates using the Thermal cyclers C1000 Touch in combination with the optical modules CFX384 (Biorad, LLEB, UAB). The amplification protocol was an initial denaturation and activation step at 50ºC for 2 min and 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s and 63ºC for 1 min. After the amplification phase, a temperature-determining dissociation step was carried out at 95ºC for 15 s, 60ºC for 15 s, and 95ºC for 15 s. Changes in gene expression in testicular samples were determined as fold-changes with respect to the saline group at each time point (24, 48 or 72 h) using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

2.7. Evaluation of sperm production

The total volume of milt collected from each male was recorded, and an aliquot was diluted 1:10 with non-activating medium (NAM; in mM: 75 NaCl, 1.5 KCl, 12.9 $MgCl_2$, 2.65 $CaCl_2$, 20 $NaHCO_3$, 4.4 glucose, 0.015 BSA, pH 7.7, 290 mOsm). The concentration of Spz was determined by loading the diluted sperm sample under a cover slip before being video-recorded for 1 second and analysed using the Integrated Semen Analysis System (ISASv1 software, Proiser, Valencia, Spain) coupled to a phase contrast microscope (Nikon Eclipse 50i, Nikon) equipped with a x20 negative phase contrast objective. Sperm count was performed in three different regions of the counting chamber to minimize miscalculations. The total amount of Spz per ejaculate was finally normalized by the weight of each fish. The measurements were carried out in duplicate for each ejaculate.

2.8. Statistical analysis

Results are expressed as the means ± SEM. Comparisons between two independent groups were made by the two-tailed unpaired Student's *t*-test. The statistical significance among multiple groups was analyzed by one-way ANOVA, followed by the Tukey's multiple comparison test, or by the non-parametric Kruskal-Wallis test and further Dunn's test for nonparametric post hoc comparisons, as appropriate. Percentages were square root transformed prior to analyses. Statistical

analyses were carried out using the GraphPad Prism v8.4.3 (686) software (GraphPad Software). In all cases, statistical significance was defined as $P < 0.05$.

3. Results

3.1. Experiment 1: effect of recombinant gonadotropins on spermiogenesis

3.1.1. rFsh and rLh increase androgen plasma levels

To monitor the correct administration and bioactivity of the recombinant hormones, the plasma levels of Fsh, Lh and 11-KT were determined by specific ELISAs. Prior to the injection with rFsh, and after acclimation at 12ºC (time 0), plasma levels of Fsh in both experimental groups were relatively low (1.45 ± 0.32 and 1.47 ± 0.40 ng ml^{-1}), and in the control group they remained low (< 2 ng ml^{-1}) throughout the experiment (Fig. 1A). However, the levels in the group treated with rFsh (18 µg kg^{-1}) for 5 weeks followed by a rLh (18 µg kg^{-1}) injection reached 17.91 ± 2.40 ng ml^{-1} 24 h after the rLh induction, and these levels decreased progressively at 48 and 72 h (10.82 ± 0.41 and 9.34 ± 1.46 ng ml^{-1}, respectively) (Fig. 1A).

As for Fsh, the plasma levels of Lh were low at time 0 (6.04 ± 0.71 and 5.40 ± 0.32 ng ml^{-1}). As expected, the group treated with rFsh showed a potent increase in the circulating levels of Lh 24 h after rLh injection (82.82 ± 8.57 ng ml^{-1}), which progressively decreased at 48 and 72 h (39.34 ± 3.36 and 21.88 ± 3.45 ng ml^{-1}) (Fig. 1B).

The changes in the plasma levels of the androgen 11-KT exhibited a similar pattern to that of the gonadotropins. These levels were low at time 0 (5.04 ± 1.24 and 5.84 ± 0.84 ng ml^{-1} in each group), and slightly increased toward the experiment in the control group (from 2.97 ± 0.52 to 10.22 ± 2,13 ng ml^{-1}), thus inversely to that observed for the Lh plasma levels in this group (Fig. 1C). According to the strong increase in plasma Lh at 24 h after rLh treatment observed in the rFsh-treated males, the 11-KT plasma levels in this group were also highly stimulated (101.41 ± 13.27 ng ml^{-1}), but the levels progressively diminished thereafter (64.30 ± 18.35 and 28.19 ± 6.67 ng ml^{-1}, at 48 and 72 h, respectively) (Fig. 1C).

3.1.2. Recombinant gonadotropins stimulate gonad growth and spermatogenesis

The treatment with rFsh followed by rLh injection clearly stimulated the testis size as indicated by the GSI of the males treated with the hormones, which was higher than that of the control fish at 24, 48 and 72 h after rLh injection (Fig. 2A). However, the GSI values in the rFsh+rLh-treated males were higher at 48 h than at 24 or 72 h after injection (Fig. 2A).

The visual examination of the testicular histology from hormone treated and non-treated males suggested that spermatogenesis and spermiogenesis was potentiated by rFsh and rLh. In this group, more Spz within the cortical part of the testis were observed with respect to the controls (Fig 2.B). This observation was confirmed by the quantification of the different germ cell types within the seminiferous tubules of the testis. The SpgA germinal stem cells represented a low percentage of the cells within the tubules at all sampling times, and their number decreased with the rFsh+rLh treatment at 24 h after rLh injection (Fig 2.C). In contrast, both the percentage of dividing SpgB and Spc increased within the tubules at both 24 and 48 h after rLh administration with respect the controls (Fig 2.C), suggesting that germ cell meiosis was stimulated in the hormone-treated group. However, the highest percentage of cells encountered within the testicular tubules of control and treated males were Spd (Fig

2.C). The majority of Spd were attached to the Sertoli cells (Spd_A), while some were observed free within the tubule lumen (Spd_F) (Fig 2.B), a typical feature of the semi-cystic spermatogenesis in the Senegalese sole. After rLh treatment, the percentage of Spd_A decreased in the rLh-treated fish with respect to the controls at all time points, whereas the occurrence of Spd_F increased only at 48 and 72 h after rLh injection (Fig 2.C). Finally, the percentage of testicular Spz was higher than the controls after 24, 48 or 72 h of rLh injection, although this percentage also slightly increased in the males treated with saline at 72 h (Fig 2.C). Altogether these data corroborated that spermiogenesis was stimulated in the males treated with the recombinant hormones.

To further confirm that the treatment with rFsh and rLh potentiated spermiogenesis, we evaluated the number of Spz within the tubules of the testicular efferent duct (ED). The histological analysis showed that the control and treated males had a similar concentration of Spz within the ED tubules, although the diameter of the tubules appeared to be higher in the males treated with rFsh and rLh with respect to that in the control fish (Fig. 3A). Determination of the tubule area confirmed that this was 5, 7 and 3 times bigger in hormone-treated fish than in the controls at 24, 48 and 72 h after rLh injection, respectively (Fig. 3B). Despite this, the concentration of Spz within the tubule was similar in controls and treated fish (Fig. 3C), and therefore the total estimated number of Spz in the ED tubules was 6-, 10- and 3-fold higher in the treated males than in the controls at 24, 48 and 72 h postinjection, respectively (Fig. 3D). The combined administration of rFsh and rLh thus enhanced the accumulation of Spz within the ED tubules, and this tended to be higher at 48 h after rLh injection.

3.1.3. rLh modulates the expression of sperm maturation-related genes

The previous data suggested that gonadotropin treatments induced the differentiation of Spz in the testis and their fast accumulation in the tubules of the ED already at 25 h after rLh injection. However, to investigate potential differences in sperm maturation after rLh induction we evaluated by qRT-PCR the level of expression of genes typically involved in teleost spermiation, such as progestin synthesis and progestin receptors (*cyp17a1*, *cyp17a2*, *cbr1* and *paqr7*), fertilization (*spag6* and *spa17*), and Spz flagellar motility (*cfap46*, *cfap54*, *cfap61* and *rsph1*). The result showed that while the expression of *cyp17a1* did not change at 24, 48 or 72 h after rLh injection, that of *cyp17a2*, *cbr1* and *paqr7* was enhanced at 48 h (Fig. 4). The other genes studied (*spag6*, *spa17*, *cfap46*, *cfap54* and *cfap61*) were also upregulated at 48 h post rLh injection, except *rsph1* for which no significant differences were detected (Fig. 4). These data therefore suggest that full maturation of sole Spz seems to occur at 48 h after rLh injection.

3.1.4. Sequential sperm production

The amount of sperm produced by males injected with the saline solution or rFsh+rLh was subsequently studied in the remaining fish from each group. To investigate whether rLh could induce several batches of Spd differentiation to Spz, in these experiments the same males were stripped at 24, 48 and 72 h after hormone injection. All fish were spermiating. In the control group, the sperm production at 24h was of $1.89 \pm 0.34 \times 10^9$ Spz kg^{-1} while it was of $3.73 \pm 0.78 \times 10^9$ Spz kg^{-1} in the rFsh+rLh treated group (~2-fold increase) (Fig. 5A). The following day, at 48h post rLh injection, the same males showed much lower sperm counts (0.55 ± 0.13 and $1.50 \pm 0.19 \times 10^9$ Spz kg^{-1} in the control and hormone-treated groups, respectively), despite the fact that the treated group exhibited 2.8-fold more sperm than the controls (Fig. 5A).

The tendency of decreasing sperm counts was confirmed by the third day of stripping (72 h post rLh injection) in both groups (0.31 ± 0.08 and 0.88 ± 0.20 x 10^9 Spz kg^{-1} in the control and hormone-treated groups, respectively), with 2.9-fold more sperm collected for the rFsh+rLh group (Fig. 5A). During the three consecutive days of collection, the accumulated total amount of sperm produced in the control and treated males reached 2.74 and 6.10 x 10^9 Spz kg^{-1}, respectively, thus being 2.2-fold higher in the males injected with rFsh+rLh than in the controls (Fig. 5B).

3.2. Experiment 2: effect of rFsh dose and temperature at spermiation

3.2.1. Gonadotropin and steroid plasma levels

In the second trial, we tested the effect of the administration of different doses of rFsh (10 or 18 µg kg^{-1}) for 5 weeks, as well as different temperatures (12º or 17ºC) at the time of rLh injection, on sperm production (Fig. 6A). To confirm the observations of the previous experiment, blood sampling and stripping of different males at 24, 48 and 72 h after rLh injection at 12ºC were only carried out in Group 6 treated with the highest dose of rFsh. For the males treated with rLh at 17ºC, blood sampling and stripping were performed only at 48 h after rLh injection based on the results of the first experiment (Fig. 6A).

As observed in the previous experiment, the plasma levels of Fsh were relatively low at time 0 (8.19 ± 2.49 ng ml^{-1}) and remained < 4 ng ml^{-1} in the controls (Groups 1 and 2) regardless of the temperature at the time of rLh injection (Fig. 6B). In contrast, males treated with 10 or 18 ug kg^{-1} rFsh (Groups 3-4 and 5-6, respectively) showed a dose dependent increase in plasma Fsh before rLh treatment, with levels reaching 34.31 ± 4.25 and 58.55 ± 3.69 ng ml^{-1} in Groups 5 (acclimated to 17ºC) and 6 (maintained at 12ºC), respectively (Fig. 6B). The levels of Fsh in these groups progressively decreased at 48 and 72 h following rLh injection, falling to 19.43 ± 1.01 and 34.32 ± 1.81 ng ml^{-1} in Groups 5 and 6, respectively (Fig. 6B).

The endogenous levels of plasma Lh in the males at time 0 (3.01 ± 0.50 ng ml^{-1}) were lower than those of Fsh and remained equally low regardless of the temperature treatment until rLh was administered (Fig. 6C). After rLh injection at 12ºC, the plasma levels of Lh markedly increased at 24 h (62.12 ± 3.56 ng ml^{-1}) to progressively decrease thereafter at 72 h (32.13 ± 2.43 ng ml^{-1}), while at 17ºC the rLh injection promoted a similar induction of plasma Lh at 48 h as at 12ºC (29.03 ± 1.36 *vs* 29.56 ± 1.74 ng ml^{-1} at 12º and 17ºC, respectively) (Fig. 6C). Curiously, males treated with the highest dose of rFsh showed the highest level of plasma Lh after rLh injection at 48 h and 12ºC, while the opposite trend was noted at 17ºC (Fig. 6C).

The 11-KT plasma levels were also fairly low at time 0 (14.78 ± 3.81 ng ml^{-1}), and after the rFsh treatment period, males at 12ºC showed higher levels of plasma androgen before rLh treatment than the controls (Fig. 6D). In contrast, the concentrations of 11-KT in males acclimated to 17ºC were not different between rFsh treated and non-treated fish (Fig. 6D), which may be related to the lower levels of plasma Fsh after the rFsh treatment in fish acclimated to 17ºC (Fig. 6B). At 12ºC, males previously treated or not with rFsh showed a similar ~3.6-fold increment of the androgen levels at 48 h after rLh injection, which decreased at 72 h, whereas at 17ºC the increase of 11-KT at 48 h was similar than that at 12ºC (Fig. 6D). Interestingly, 48 h after rLh injection at either 12º or 17ºC the levels of 11-KT were not affected by the previous treatment of males with rFsh, unlike that observed for Lh (Fig. 6D).

3.2.2. rFsh and rLh-induced sperm production is enhanced at high temperature

Sperm production was evaluated at 48 h after rLh injection (Groups 1-5), or at 24, 48 and 72 h post rLh treatment using different subgroups of males from Group 6. As observed in the experiment 1 all fish were spermiating. At 12ºC, males treated with the highest dose of rFsh (18 µg kg^{-1}) produced 0.56 ± 0.04 $x10^9$ Spz kg^{-1} at 24 h after rLh injection, which was ~6-fold higher than that of the controls (0.09 ± 0.04 $x10^9$ Spz kg^{-1}) at 48 h postinjection (Fig. 7). When the rFsh-treated fish were stripped at 48 h, the sperm count was almost doubled (0.95 ± 0.18 $x10^9$ Spz kg^{-1}) with respect to the fish spermiated at 24 h, representing a ~11-fold increase with respect to the control group, whereas at 72 h sperm production dropped (0.44 ± 0.13 $x10^9$ Spz kg^{-1}) (Fig. 7). As expected, males treated with the low dose of rFsh (10 µg kg^{-1}) and injected with rLh at 12ºC were less effective in producing sperm at 48 h (0.36 ± 0.06 $x10^9$ Spz kg^{-1}) (Fig. 7). However, this was not the case when males were acclimated to 17ºC before rLh injection (Groups 4 and 5), since in these groups the sperm produced by fish previously treated with 10 or 18 µg kg^{-1} rFsh was similar at 48 h after rLh injection (1.06 ± 0.30 and 0.87 ± 0.21 $x10^9$ Spz kg^{-1}, respectively), and as high as in males treated with 18 µg kg^{-1} of rFsh and rLh at 12ºC (Fig. 7).

4. Discussion

In the present study, two different experiments were carried out in which rFsh was administered during 5 consecutive weeks under a controlled temperature of 12ºC. Such a low temperature seems to be positive to potentiate spermatogenesis in Senegalese sole males, since it correlated with a strong increment in the GSI and the total production of sperm as found here and in previous studies (García-López et al., 2006; Chauvigné et al., 2017, 2018). In both experiments of the present study, the endogenous basal levels of Fsh and Lh in plasma before rFsh treatment were low (~5 ng ml^{-1}), suggesting that the acclimation periods of the fish to the low temperature were efficient. The administration of rFsh at 12ºC may also be beneficial to increment the stability of the hormone in plasma. Indeed, in the present study, the plasma levels of Fsh before rLh injection reached ~35 or ~60 ng ml^{-1} in males treated with 10 or 18 µg kg^{-1} rFsh at 12ºC, respectively, while they dropped to ~17 or ~35 ng ml^{-1} when fish were acclimated to 17°C.

The plasma levels of Lh in males treated with 10 or 18 µg kg^{-1} rFsh showed however a different trend depending on the temperature, which has not been previously observed. Thus, after rLh injection at 12ºC the plasma levels of Lh in males increased in a dose dependent manner with the previous dose of rFsh received, whereas at 17ºC a decrease of the Lh levels with the rFsh dose was noted. These data could reveal differences in hormone kinetics at the temperatures tested, or a possible feedback regulation on Lhβ expression and secretion by the pituitary induced indirectly by testicular steroids produced in response to rFsh, or through dopamine regulatory mechanisms in the brain triggered by the hormone (Yaron and Levavi-Sivan, 2011). Future studies will be necessary to investigate whether these mechanisms can modulate the rLh induction of spermiation in Senegalese sole.

The combined treatment of rFsh and rLh raised the plasma levels of the androgen 11-KT, which confirmed the strong bioactivity of the recombinant gonadotropins (Chauvigné et al., 2017, 2018). However, the rLh treatment appeared to be more potent than rFsh at inducing androgen secretion, as observed in other fish species (Kazeto et al., 2008; Yom-Din et al., 2016). The rLh-stimulated 11-KT synthesis also resulted in an increase of the GSI after 48 h of rLh injection, reflecting the growth of the testis during the treatment. In the present work, the GSI approximately doubled with respect

to the controls after 5 weeks of rFsh treatment and a single rLh injection, a result comparable to that found in F1 pubescent sole males treated with the same dose of rFsh for 9 weeks under natural temperature (from 15º to 11ºC) (Chauvigné et al., 2017). This again suggests that an acclimation to low temperature favours testis growth and spermatogenesis in sole.

Histological analysis of the different cell types in the testis revealed an accumulation of SpgB in the seminiferous tubules at 24 h after rLh injection, which was concomitant with a decreased percentage of SpgA, which is in agreement with the differentiation and proliferation of SpgB at the onset of spermatogenesis in teleosts (Schulz et al., 2010). A higher occurrence of Spc was also found in the hormone treated fish, indicating that the treatment with rFsh and rLh induced an entry of Spg into meiosis. No effect was observed on the number of SpgA, SpgB or Spc after 72 h, suggesting that cells already differentiated to haploid spermatids. Similar results were previously described for Senegalese sole (Chauvigné et al., 2017), as well as other in other teleosts in which species-specific recombinant gonadotropins have been employed (Peñaranda et al., 2018, Molés et al., 2020). At all sampling times, the percentage of immature Spd_A was decreasing while that of mature Spd_F, as well as the number of Spz, increased in the tubules at 48 and 72 h after rLh induction, as observed in our previous study (Chauvigné et al., 2017). These data thus reveal an active spermiogenesis controlled by gonadotropins, which was corroborated by the increment in the number of Spz in the ED. Therefore, as previously reported in Senegalese sole males (Chauvigné et al., 2017), recombinant gonadotropin-based hormone therapies appear to be effective to promote spermatogenesis and spermiation in this species.

It is known that C_{21} steroids (progestins) are active players in the process of spermiation in teleosts (Scott et al., 2010). Progestins, such as 17α,20β-dihydroxypregn-4-en-3-one (17,20βP) or 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) are known maturation inducing steroids in male and female teleost gametes (Scott et al., 2010). Progestins can induce spermiation, increase milt production under the control of Lh, and stimulate Spz motility (Scott et al., 2010; Vizziano et al., 1996; Yueh and Chang, 1997; Tubbs and Thomas, 2008; Tenegu et al., 2020). In Senegalese sole, however, previous studies have reported that the plasma levels of 17,20βP are almost undetectable at the time of spermiation (Garcia-López et al., 2006; Agulleiro et al., 2007). In contrast, free and sulphated 17,20βP and its metabolites are readily detectable in males in which spermatogenesis is enhanced by treatment with GnRHa in combination with 11-ketoandrostenedione (Agulleiro et al., 2007). This suggests that sulphated or glucuronidated and/or 5β-reduced 17,20βP metabolites may be the active 'spermiation-inducing' hormones in Senegalese sole as in other flatfishes (Agulleiro et al., 2007; Scott et al., 2010).

Therefore, as a proxy to monitor the process of sperm maturation after rLh treatment, we investigated the expression of various genes related to progestin synthesis and function at 24, 48 and 72 h after rLh injection. In the testis, progestins are synthetized in the interstitial Leydig cells from their precursor progesterone, which is metabolised to 17-hydroxyprogesterone (17-P) by the Cyp17a1 enzyme through its 17α-hydroxylase activity (Kazeto et al., 2000). The Cyp17a1 has also lyase activity, converting 17-P to androstenedione, the immediate precursor of testosterone, which is also the precursor of estrogens and 11-KT in male fish. In salmonids and possibly in other teleosts, another Cyp17a1-related enzyme, termed Cyp17a2, which exhibits hydroxylase activity only, as well as the Cbr1, are upregulated during spermiation, thus driving the accumulation of 17-P and further conversion to 17,20βP (Zhou et al., 2007; Sreenivasulu et al., 2012). Therefore, a shift in the ratio between the two Cyp17a

enzymes, or alternatively the inhibition of the Cyp17a1 lyase activity by progestins themselves, may lead to the synthesis of progestins rather than androgens (Barry et al., 1990; Tenugu et al., 2020). According to this model, we observed that the *cyp17a1* expression levels did not vary following rLh induction, while those of *cyp17a2* and *cbr1* increased more at 48 h after rLh injection, suggesting a shift to progestin synthesis in the testis at the time of maximum spermiation. Progestins can act on Spz through the membrane progestin receptors, such as Paqr7 (Thomas et al., 2009), and in our study we also detected the highest level of the corresponding *paqr7* transcripts at 48 h after rLh treatment. These data therefore suggest that full maturation of Spz in the ED of the testis possibly occurs at 48 h post rLh induction. This conclusion is supported by the expression of other genes potentially involved in sperm motility, such as *cfap46*, *cfap54* and *cfap61* (Linck et al., 2016; McKenzie et al., 2020; Huang et al., 2020; Liu et al., 2021), and sperm fertilization competence, such as *spag6* and *spa17* (Liu et al., 2019; Instaqui et al., 2017), which were also upregulated in the testis at 48 h after rLh injection.

The sequential stripping of males at 24, 48 and 72 h following rLh treatment revealed that sperm counts, while remaining higher than in the controls, were progressively decreased from 24 to 72 h post injection, suggesting that the rLh induced the recruitment of only one batch of Spd into Spz differentiation and maturation. This observation was confirmed in the second experiment, in which males stripped at 48 h showed more ejaculated sperm than males sampled at 24 or 72 h after rLh injection. These data agree with the asynchronous type of spermatogenesis described in Senegalese sole (García-López et al., 2005, 2006), and could be the result of a negative feedback mechanism on Spz differentiation occurring in the testis. Although the nature of these mechanisms are yet unknown, previous studies in sole have identified that the maturation of Spds is associated with the translation of the Lh receptor in these cells and their release to the lumen of the seminiferous tubules, where they will differentiate to Spz in response to Lh (Chauvigné at al., 2014ab). The investigation of the molecular regulation of the Lh receptor in immature Spd_A will therefore be of interest to elucidate the endocrine mechanisms controlling spermiation in Senegalese sole.

Finally, we investigated the effect of a rise in temperature prior to the rLh administration on sperm production. The aim of this experiment was to replicate the conditions of maximum spermiation in the wild, which occurs in spring when temperature is around 16-18°C (Cerdà et al., 2008, García-López et al, 2006, Guzmán et al., 2009). At 12ºC, sperm production at 48 h after rLh treatment was increased in a dose-dependent manner with the dose of rFsh previously administered, but at 17ºC we found that rLh injection of males previously treated with a low dose of rFsh resulted in a similar production of sperm at 48 h than that observed in males treated with a high dose of rFsh and rLh at 12ºC. Whether this observation is the result of more Spd_A being recruited into maturation at 17ºC, or of a faster process of Spd_F differentiation to Spz, remains to be studied. In addition, in the present study we did not measure sperm production at 24 h after rLh injection at 17ºC, and therefore it is possible that under these conditions production of sperm at this time can be similar to that at 48 h. Nevertheless, our results suggest that maximum spermiation was obtained under the hormone doses and conditions employed in the present study. Future research should be conducted to synchronize the testis to accumulate more SpdF in the testis prior to the rLh injection, which might increase the quantity of sperm collected.

In conclusion, we confirm the efficiency of a dual rFsh and rLh hormone therapy to enhance testis growth, spermatogenesis and spermiation in Senegalese sole. Our data also suggest that 48 h after rLh injection is the time that assures maximum production of

mature Spz. In addition, we report that using a low dose of rFsh for 5 consecutive weeks at 12ºC is very efficient in terms of sperm production if further rLh treatment is given at 17ºC, which could be of interest to reduce the economic cost of using rFsh and rLh in a commercial hatchery. Altogether, the present study therefore proposes a more refined protocol for recombinant gonadotropin-based hormone therapies to promote sperm production in F1 Senegalese sole males.

Author statement

Chauvigné, F: Conceptualization, Investigation, Methodology, Data curation, Writing-Original draft preparation, Supervision, Funding acquisition. **Lleberia, J:** Investigation, Methodology. **Vilafranca, C:** Investigation. **Rosado, D:** Investigation. **Martins, M:** Investigation. **Silva, F:** Investigation. **Gonzalez-López, WA:** Investigation. **Ramos-Júdez, S:** Investigation. **Duncan, N:** Investigation, Writing-Reviewing and Editing. **Giménez, I:** Conceptualization, Methodology, Writing-Reviewing and Editing. **Blanquet, I:** Conceptualization, Investigation, Methodology, Writing-Reviewing and Editing, Supervision, Funding acquisition. **Cerdà, J:** Conceptualization, Investigation, Methodology, Data curation, Writing-Reviewing and Editing, Supervision, Funding acquisition, Project administration.

Declaration of Competing Interest

The recombinant gonadotropins employed in this study were produced by the biotech commercial company Rara Avis Biotec, S. L. (Valencia, Spain). There are no other competing interest to declare.

Acknowledgments

The authors thank the technical staff from IRTA and Safiestela Ltd. for their help during the maintenance and sampling of fish. This work was supported by the Spanish Ministry of Science and Innovation (MINECO) (AGL2017-84013-R to F.C.), the European Commission Portugal 2020 Program (European Regional Development Fund to I.B.), and the CERCA Programme (Government of Catalonia). Participation of F.C. was funded by a “Ramon y Cajal” contract (RYC-2015-17103, MINECO). Participation of S.R. and W.G. was funded, respectively, by a PhD grant from AGAUR Catalonian Agency for the Management of Universities and Research Grants (Government of Catalonia, Spain) co-financed by the European Social Fund, and a PhD grant from the National Board of Science and Technology (CONACYT, Mexico).

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Figures

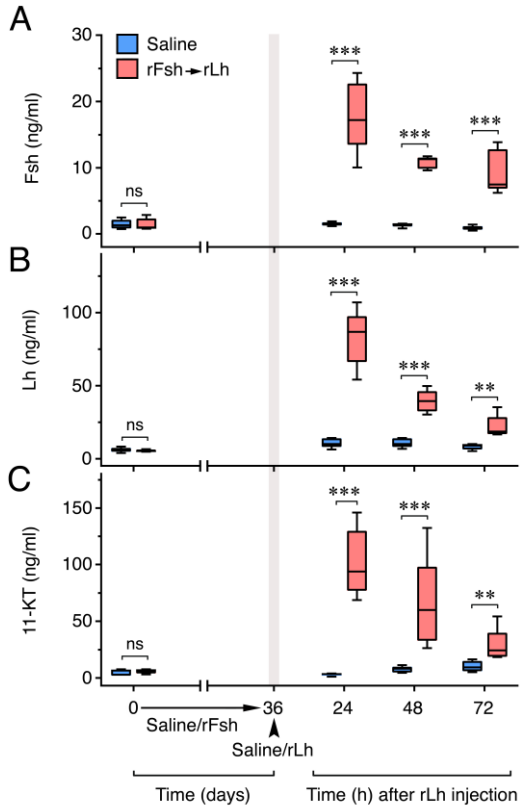

Fig. 1. Gonadotropin and androgen circulating levels in Senegalese sole males treated with rFsh and rLh. Plasma levels of Fsh (A), Lh (B) and 11-KT (C) in males before rFsh (18 µg kg^{-1}) treatment (day 0), and at 24, 48 and 72 h after saline (control) or rLh (18 µg kg^{-1}) intramuscular injection following a weekly treatment with saline or rFsh for 5 weeks. Data (n = 5 fish) are box and whisker plots and were statistically analyzed by the Student's t-test at each time point as indicated in brackets (**, $P < 0.01$; ***, $P < 0.001$).

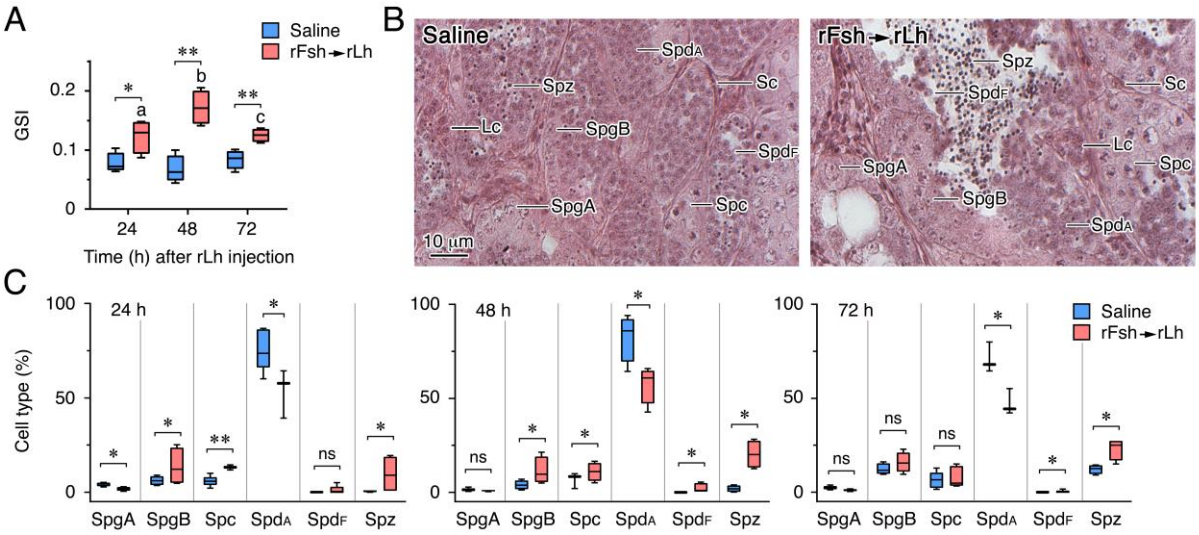

Fig. 2. Gonad weight and testicular development of males treated with rFsh and rLh. (A) GSI of males, previously treated with rFsh (18 µg kg^{-1}) for 5 weeks at 12ºC, at 24, 48 and 72 h after saline or rLh (18 µg kg^{-1}) injection at the same temperature. (B) Representative photomicrographs of histological sections from the cortical region of the testis stained with hematoxylin and eosin after 48 h of treatment with saline or rLh. (C) Percentage of germ cells in the seminiferous tubules in the testis of fish at 24, 48 and 72 h after treatment with saline or rLh. SpgA, spermatogonia type A; SpgB, spermatogonia type B; Spc, spermatocyte; Spd_A, spermatid attached to Sertoli cells; Spd_F, spermatid free in the tubule lumen; Spz, spermatozoa. In A and C, data (n = 5 fish) are box and whisker plots and were statistically analyzed by the Student's t-test at each time point or cell type, or as indicated in brackets (*, $P < 0.05$; **, $P < 0.01$). In A, statistically significant data among the rFsh+rLh-treated males at different times are indicated by a different superscript on top of each box (one-way ANOVA, $P < 0.05$).

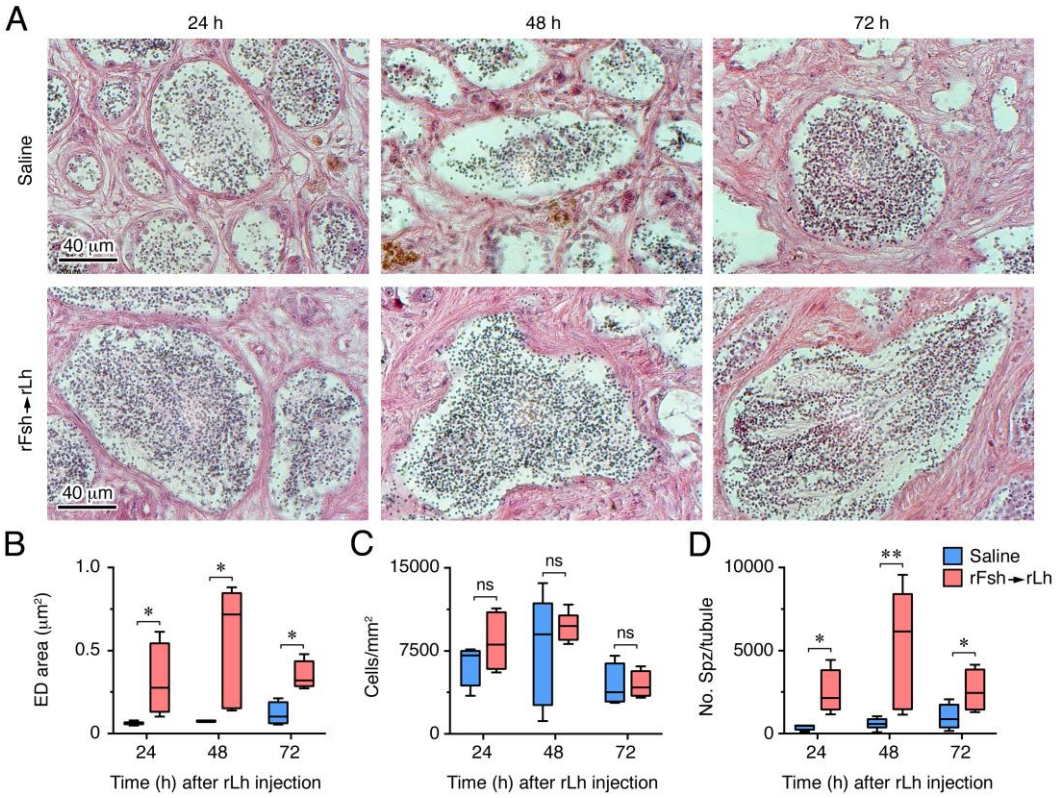

Fig. 3. Accumulation of spermatozoa in the testicular efferent duct (ED) of males treated with rFsh and rLh at 12ºC. (A) Photomicrographs of histological sections from the ED stained with hematoxylin and eosin from males at 24, 48 and 72 h after saline or rLh treatment. (B-D) Area of the ED lumen (B), density of spermatozoa (C) and number of spermatozoa per ED tubule (D) after saline or hormone treatment. In B-D, data (n = 5 fish) are box and whisker plots and were statistically analyzed by the Student's t-test at each time point (*, $P < 0.05$; **, $P < 0.01$).

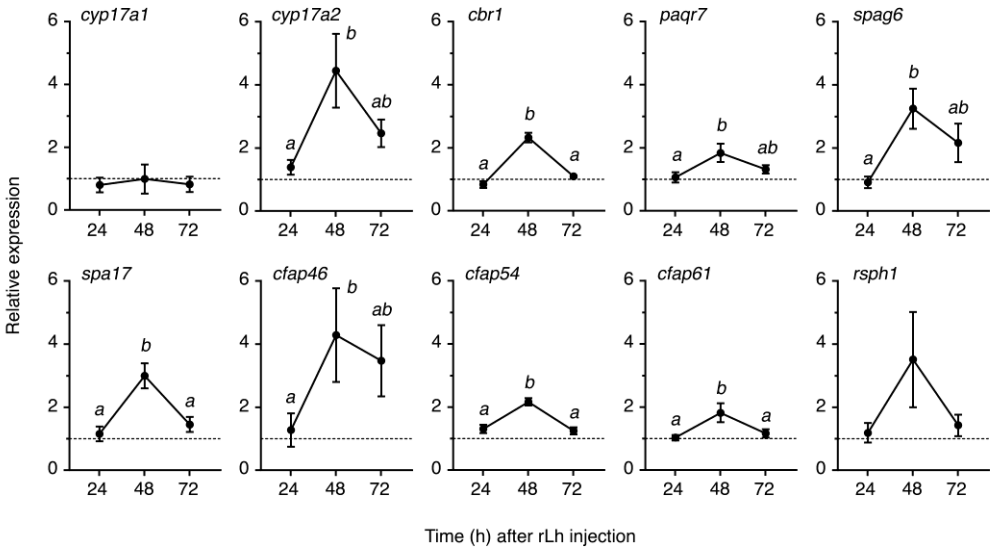

Fig. 4. Changes in the expression of testicular genes related to progestin function, flagellar motility and fertilization, in males treated with rFsh and rLh at 12ºC. Values are the relative mean expression levels of different genes normalized to the β-actin gene after 24, 48 and 72 h of rLh treatment expressed as fold-changes with respect to the control group (saline injected) at each time point. Dashed line at 1 indicates no change with respect to the controls. Data are the mean ± SEM (n = 4 fish), and values with different superscript are significantly different (one-way ANOVA, $P < 0.05$).

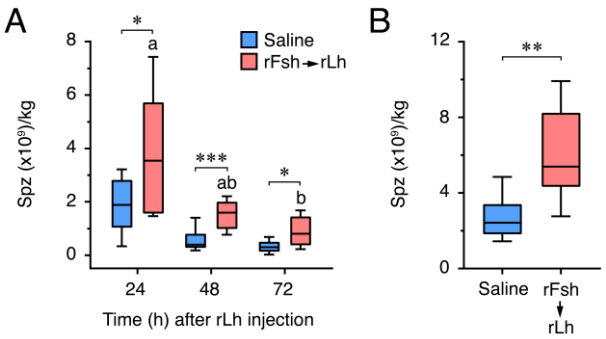

Fig. 5. Sperm production by males treated with rFsh and rLh at 12ºC. (A) Mean amount of sperm, normalized to the weight of fish, produced by the same males at 24, 48 and 72 h after saline or rLh injection. (B) Total sperm produced by each group during three days after the treatments. Data (n = 10 fish) are box and whisker plots and were statistically analyzed by the Student's t-test as indicatd in brackets (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). In A, statistically significant data among the rFsh+rLh-treated males at different times are indicated by a different superscript on top of each box (one-way ANOVA, $P < 0.05$).

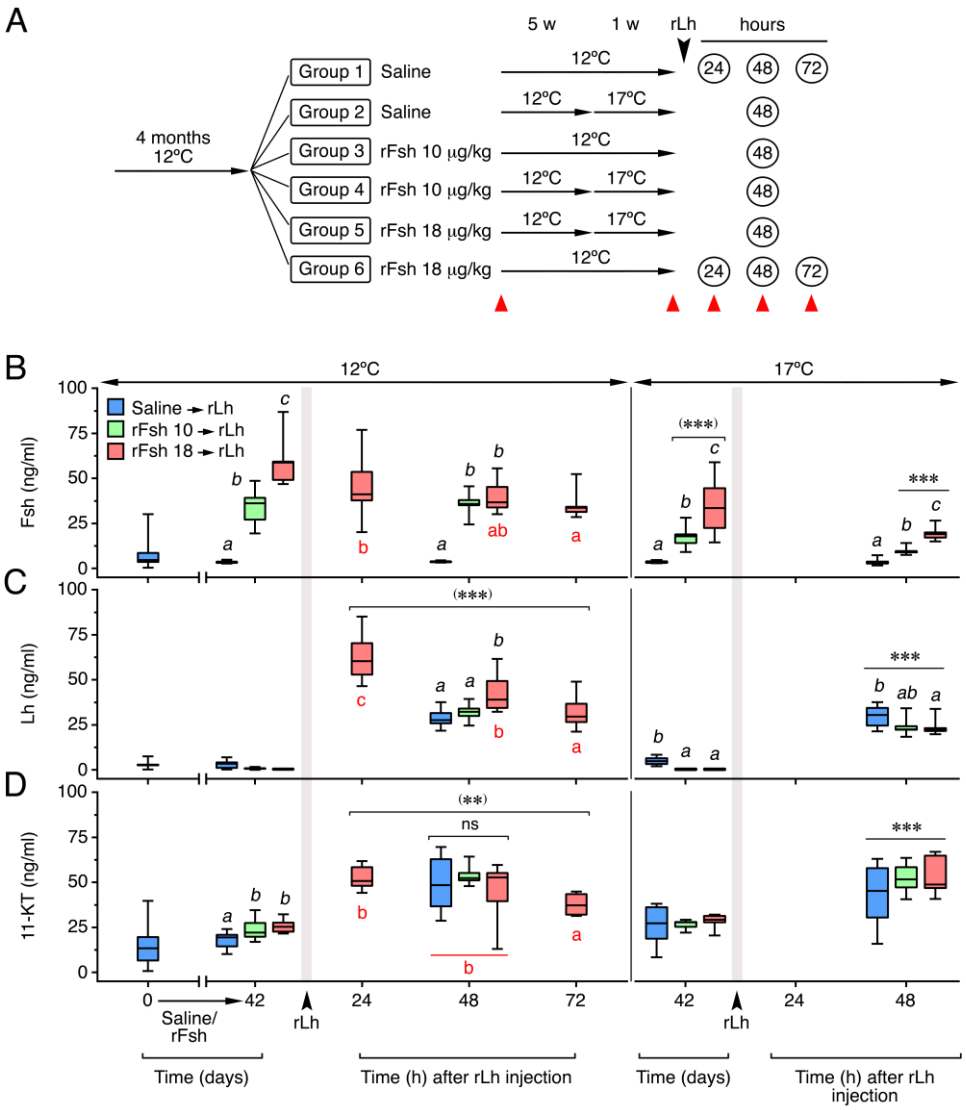

Fig. 6. Gonadotropin and androgen plasma levels in Senegalese sole males treated with two doses of rFsh and with one rLh dose at two different temperatures. (A) Schematic representation of the experimental setup. (B-D) Concentration of Fsh (B), Lh (C) and 11-KT (D) were measured at day 0 (before rFsh treatment), after the saline or rFsh treatment with 10 or 18 µg kg^{-1} (rFsh 10 and rFsh 18, respectively) for 5 weeks plus one more week at 12 or 17ºC (day 42), and at 24, 48 and 72 h after rLh (18 µg kg^{-1}) injection at 12 or 17ºC. Note that in this case control fish were also treated with rLh at day 42. Data (n = 12 fish) are box and whisker plots and were statistically analyzed by one-way ANOVA. Bars with different superscript within a time point (black color) or amongst the times after rLh injection (red color) at 12ºC are significantly different ($P < 0.05$). The asterisks in parenthesis indicate data significantly different with respect to groups treated with rFsh at 12ºC before rLh injection, whereas asterisks without parenthesis indicate differences with respect to groups maintained at 17ºC before rLh treatment (**, $P < 0.01$; ***, $P < 0.001$).

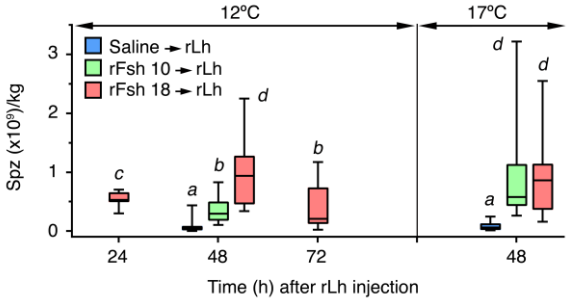

Fig. 7. Sperm production by males treated with two doses of rFsh and with one rLh dose at two different temperatures. Data represent the mean amount of sperm, normalized to the weight of fish, produced by different males at 24, 48 and 72 h after rLh injection. Note that control and 10 µg/kg rFsh treated fish were spermiated only at 48 h. Data (n = 12 fish) are box and whisker plots and were statistically analyzed by one-way ANOVA. Bars with different superscript are significantly different ($P < 0.05$).